Characterization of Biosurfactants of Bacillus sp and its Application as Antibiofilm Agents

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Abstract:

Biofilms are complex assemblages of microbial cells enclosed in a self synthesized polymeric matrix. Bacteria growing as abiofilm remain a significant challenge in biomedical field, growing on abiotic material such as catheters and prosthesis as they tend to be more tolerant to antimicrobial treatments. Current microbial inhibition strategies based on planktonic bacterial physiology have been known to have limited efficacy on the growth of biofilm communities. This problem is further intensified by the emergence of increasingly resistant strains. All aspects of biofilm measurement monitoring, dispersal, control and inhibition are becoming issues of increasing importance. Biosurfactants have proved their potential in both clinical and hygienic sectors due to their ability to disperse microbial biofilms. The surface and interfacial tension reducing properties of biosurfactants make them suitable candidates as dispersing agent. In addition to this, due to their specificity, low toxicity, high biodegradability, widespread applicability and effectiveness at extremes of pH and temperature, a considerable amount of research is being focused on biosurfactant producing bacteria, fungi, yeast and actinomycetes. Bacillus is the dominant groups of bacteria found in almost all environments and have proved their prolific potential in production of biotechnologically relevant molecules. In this study, we proposed to characterize the biosurfactants produced by Bacillus sp and examine their role in disruption or inhibition of microbial biofilms.

Introduction:

Microbial surfactants or biosurfactants are low molecular weight, amphipathic, surface active compounds produced by microorganisms. Biosurfactants are characteristic of reducing the surface and interfacial tensions between individual molecules at the surface and interface respectively in both aqueous solutions and

hydrocarbon mixtures. Most biosurfactants are diverse, complex molecules, comprising of different structures that include glycolipids, lipopeptides, lipoproteins, fatty acids, phospholipids, neutral lipids, polymeric and particulate biosurfactants. Biosurfactants have received enormous attention because of their versatile applications in petroleum industry, environmental remediation, food processing, agro industry, cosmetics and pharmaceuticals [Banat et al., 2000]. They are highly preferred over their synthetic counterparts due to their reduced toxicity, higher biodegradability, ecological acceptability and specific activity in wide range of temperature, pH and salinity [Lima et al., 2010]. Besides their regular applications in bioremediation and hydrocarbon degradation, they are employed in formation of ion channels in lipid membranes and inhibition of fibrin clot formation. They are also explored for their antitumor, antiviral and anti-mycoplasmic activities [Cao et al., 2009; Remichkova et al., 2008]. In recent years, biosurfactants are considered as iron chelating agents [Bhosale et al., 2015] and potential drug candidates and looked up for their explicit biomedical applications such as antibacterial, antifungal, antiviral and antiadhesive agents against several drug resistant pathogens [Remichkova et al., 2008; Gudina et al., 2010; Kim et al., 2010; Kiran et al., 2010].

Biofilms are complex aggregation of bacteria enfolded in an extracellular polymeric substance (EPS) which forms a protective barrier for the indwelling microbes from the hostile environment. The bacterial pathogens that adapt biofilm mode of growth are several fold resistant to bactericidal agents than their planktonic counterparts. There are reports which demonstarte that antibiotic resistance is attributed to the complex nature of biofilms formed by the microbes in vivo [Kalia, 2014; Kumar et al., 2011]. Antibiotics were believed to be the remedy for all ailments caused by bacterial pathogens, until the incidence of drug resistance emerged. Continuous and indiscriminate usage of antibiotics have paved way for the development of antibiotic resistant bacteria and few which were resistant to multiple antibiotics. Antibiotics have become ineffective in controlling the virulence of bacterial pathogens [Kumar et al., 2011; Kalia et al., 2014; Chu et al., 2013]. Hence, novel antimicrobial agents which selectively reduce the virulence of pathogens rather than inducing antimicrobial resistance are the need of the hour. Antibiofilm agents are one such a kind which selectively eradicate the persistent biofilms thereby facilitate

the diffusion of antibiotics into the complex biofilm ambience [Chibber et al., 2013; Kalia, 2013]. Identification and characterization of new biosurfactants and antibiofilm agents with profuse biological activities will aid mankind to combat recurrent infections caused by drug resistant biofilm forming pathogens.

Though many bacterial species are reported as the promising source for the isolation of biosurfactant, there are few reports which elucidated in depth application of biosurfactants as antibiofilm agents. Bacillus spp. are main workhorses for biotechnological applications. Their products are in the GRAS list (generally regarded as safe) of US Food and Drug Administration (USFDA) and hence are regarded harmless. They produce a variety of products including extracellular enzymes, biopolymers, biosurfactants, biopesticides, and so forth from renewable resources and are ecofriendly. Hence, it can be expected that Bacillus sp isolated from different ecosystems can produce novel biosurfactants with excellent antibiofilm activity. Bacillus sp are an excellent source of novel biomolecules due to their ubiquitous presence in species richness and the chemical uniqueness of the metabolites produced by Bacillus sp. With this background, we proposed in the present study, screening of Bacillus sp, for biosurfactant production and evaluation of biosurfactants for their antibiofilm activity with the hope that biomolecules from such novel bacteria will be of new-fangled type.

Review of Literature:

Moryl et al., (2015) has studied the antimicrobial effects of lipopeptide biosurfactants from surfactin, iturin and fengycin families obtained from *Bacillus subtilis* I'1a strain, on uropathogenic bacteria, also included the effects of planktonic growth, process of biofilm formation and dislodging.

Radhika et al., (2014) reported biosurfactant production and its optimization by using RSM from a petroleum reservoir isolate *Bacillus amyloliquifaciens* USTBb. Also the characterization of produced biosurfactant was reported by using FTIR and EDS to determine the functional group and compositional analysis.

Xia et al., (2011) isolated three biosurfactant producing bacteria from reservoir formation water, *Bacillus subtilis, p. aeruginosa* and *R. erthropolis* from these isolates three biosurfactants were produced by using crude oil as a carbon source and it was observed that *P. aeruginosa* has potential to produce biosurfactant which is more effective in resistance and stability than rest of the two which has emulsification index of 80% for crude oil, reduced the surface tension from 71.2-22.6mN/M and also showed the oil recovery of 14.3% after flooding with biosurfactant.

Yakimov et al ., (1995) reported that Strain BAS50, isolated from a petroleum reservoir at a depth of 1,500 m and identified as *Bacillus licheniformis*, grew and produced a lipopeptide surfactant when cultured on a variety of substrates at salinities of up to 13% NaCl. Surfactant production occurred both aerobically and anaerobically and was optimal at 5% NaCl and temperatures between 35 and 45 ºC. The biosurfactant, termed lichenysin A, was purified and chemically characterized. Purified lichenysin A decreases the surface tension of water from 72 mN/m to 28 mN/m and achieves the critical micelle concentration with as little as 12 mg/liter, characterizing the product as a powerful surface-active agent that compares favorably to others surfactants. The antibacterial activity of lichenysin A has been demonstrated. A strain of *Bacillus subtilis* was able to grow and produce a biosurfactant on 2% sucrose at 45°C.

Jehan et al., (2015) reported the production of biosurfactant by *Leuconostoc mesenteroides ssp. cremoris* isolated from raw cow's milk was studied. Extraction of extracellular and cell-bound biosufactant was partially purified by cold acetone precipitation. The weight of them was determined and the surface activity of each of them was studied. The results showed that extracellular biosurfactant reached to 10.8 gram/1.2 dry cells/liter and 0.34 gram/1.2 dry cells/liter for cell-bound biosurfactant. Partially purified extracellular and cell-bound biosurfactant showed surface activity with oil displacement diameter 10mm. Antibacterial and antibiofilm effect of partially purified biosurfactant were evaluated against bacterial isolates including *Escherichia coli, Proteus mirabilis, Pseudomonas aeruginosa, Staphylococcus epidermidis* and *Staphylococcus aureus* isolated from catheters.

Padmavati and Pandian (2014) reported that Coral Associated Bacteria (CAB) (N=22)isolated from the mucus of the coral *Acropora digitifera* were screened for biosurfactants using classical screening methods; hemolysis test, lipase production, oil displacement, drop collapse test and emulsifying activity. Six CAB (U7, U9, U10, U13, U14, and U16) were found to produce biosurfactants and were identified by 16S ribosomal RNA gene sequencing as *Providencia rettgeri*, Psychrobacter sp., *Bacillus flexus, Bacillus anthracis, Psychrobactersp.*, and *Bacillus pumilus* respectively. Their cell surface hydrophobicity was determined by Microbial adhesion to hydrocarbon assay and the biosurfactants produced were extracted and characterized by Fourier Transform Infrared spectroscopy. Since the biosurfactants are known for their surface modifying capabilities, antibiofilm activity of positive isolates was evaluated against biofilm forming *Pseudomonas aeruginosa* ATCC10145. Stability of the active principle exhibiting antibiofilm activity was tested through various temperature treatments ranging from 60 to100 $^{\circ}$C and Proteinase K treatment. CAB isolates U7 and U9 exhibited stable antibiofilm activity even after exposure to higher temperatures which is promising for the development of novel antifouling agents for diverse industrial applications.

Objectives of Research:

- Isolation of Bacillus species from different environments.
- Screening of isolated Bacillus sp for biosurfactant production potential.
- Evaluation of biosurfactant producing Bacillus sp for antibiofilm activity.
- Identification of promising isolate at molecular level.
- Production optimization and purification of biosurfactant.
- Characterization of biosurfactant.
- Quantification of its antibiofilm activity.

Methodology to be adopted:

1. The environmental samples including soil and water from agricultural fields, oil contaminated sites, hot springs and marine environments will be collected.

2. Bacillus sp. Isolation will be carried out on different nutrient medium. Screening of isolated Bacillus sp will be carried out by using several methods of biosurfactant screening viz. drop collapse assay, oil spreading assay, haemolysis test, emulsification assay, blue agar plate test, MATS test.

3. Screening of potential biosurfactant producing Bacillus sp for antibiofilm activity will be carried out by using EPS test, CV staining test, Congo red assay.

4. By using a specified medium production of biosurfactant will be carried out and purification of produced biosurfactant will be done by using analytical methods.

5. For the characterization of produced biosurfactant techniques like TLC, FTIR, NMR, will be performed.

6. Quantification of biofilm inhibitory potential of biosurfactants

Importance of study or Society application:

- **International status:**

Quinn et al., (2013) has observed that rhamnolipids and some surface active plant oil extracts have significant role in complex biofilm inhibition and also can act as adjuvants enhancing selected antibiotics microbial inhibitors. Dos et al., (2014) has reported the anti-biofilm capacity of biofilm producing bacterium *Klebsiella pneumoniae* on several gram positive bacteria and also to some gram negative Bacilli. Ceresa et al., (2015) has recently reported biosurfactant from *Lactobacillus brevis* CV8LAC which also ahs antibiofilm activity upto 90%, and has shown antifungal activity upto 60% when used for pre-coating of silicone elastometric disks.

- **National Status:**

Joshi-Navare and Prabhune (2013) reported the synergistic effects of sophorolipid biosurfactant with selected antibiotics. Pradhan et al., (2014) reported a new glycolipid obtained from *Lysinibacillus fusiformis* S9 with potential anti-biofilm activity against pathogenic strains *E.coli* and *S. mutans* with no effects on microbial cell viability.

- **Significance of the study:**

a. This study will be helpful in enhanced production of biosurfactant The present study may contribute to isolation of new Bacillus sp contributing in production of biosurfactant with antibiofilm capacity.
b. The present study will be helpful in determination of mechanism of biosurfactant action against bacterial biofilms.

c. The present study will be helpful in production of biosurfactant which will contribute in recent drug screening program to act against drug resistant bacteria.

Proposed work Plan:
Year-wise Plan of work and targets to be achieved:

Year	**Target to be achieved**
First Year (2016-2017)	✓ Reference collection ✓ Sample collection ✓ Isolation of thermophilic Bacillus sp ✓ Screening of isolates for biosurfactant production and antibiofilm potential
Second Year (2017-2018)	✓ Production of biosurfactants ✓ Characterization of produced biosurfactants ✓ Quantification of its antibiofilm activity ✓ Compilation of data for thesis writing and publication ✓ Submission of thesis to the university

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